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# Baseline cortisol measures and developmental pathways of anxiety in early adolescence

Greaves-Lord K, Huizink AC, Oldehinkel AJ, Ormel J, Verhulst FC, Ferdinand RF. Baseline cortisol measures and developmental pathways of anxiety in early adolescence.

**Objective:** This study investigated whether baseline cortisol measures predicted future anxiety, and compared cortisol values of groups with different developmental pathways of anxiety.

**Method:** Cortisol levels were assessed in 1768 individuals (10–12 years). Anxiety levels were assessed at the same age and 2 years later.

**Results:** Cortisol measures did not predict future anxiety levels. Individuals with persistent anxiety problems did not show higher morning cortisol levels than those with persistently low, decreasing, or increasing anxiety levels. Instead, individuals with persistently high anxiety levels showed significantly lower evening cortisol levels than all other individuals. Further, participants with increasing anxiety levels showed higher morning cortisol levels (area under the curve; AUC) than individuals with persistently low anxiety levels.

**Conclusion:** The extent to which the HPA-axis – by itself – plays a role in the aetiology of anxiety is questionable. Interactions of the HPA-axis with other biological or environmental factors may be more important.

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Key words: cortisol; anxiety; adolescence; longitudinal survey

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## Significant outcomes

- Baseline cortisol measures did not predict anxiety levels two years later.
- Individuals with persistent anxiety problems did not show higher morning cortisol levels [area under the curve (AUC)] than all other individuals. Instead, they showed significantly lower evening cortisol levels.
- Participants with increasing anxiety levels showed higher morning cortisol (AUC) levels than individuals with persistently low anxiety levels.

## Limitations

- Only baseline cortisol levels were assessed, reactivity to a psychological stressor was not assessed.
- The influence of daily stresses or stressful life-events was not measured.
- Cortisol was collected at home, which may have limited the reliability of the time points at which cortisol samples were collected.

## Introduction

The onset of adolescence is characterized by high levels of anxiety (1–3). Since these problems result in considerable suffering and social impairment (4), it is important to investigate aetiological mecha-

nisms and putative biological markers to identify individuals at risk. One of the biological systems that may play a role in anxiety is the hypothalamic–pituitary–adrenal (HPA) axis. The HPA-axis is activated by stressful stimuli. Under stress, hypothalamic production of corticotropin-releasing

hormone (CRH) increases, which stimulates the pituitary release of adrenocorticotropin hormone (ACTH). As a consequence, cortisol secretion from the adrenal cortex increases. HPA-axis activity not only changes as a result of stress, but also fluctuates during normal daily activities. In the morning, as a result of waking up, cortisol levels rise, reaching a peak after approximately half an hour [cortisol awakening response (5, 6)]. After this peak, cortisol levels begin to decrease, and continue to decrease during the day.

Kagan et al. (7) have suggested that some children are susceptible to developing anxiety problems, because of a low threshold for HPA-axis activation. In these children, stimuli would more easily increase HPA-axis activity, resulting in relatively high cortisol concentrations. In time, these children would show withdrawn, anxious behaviour, because they try to avoid the unpleasant stress reaction to stimuli. One could therefore expect relatively high cortisol levels in anxious individuals. Findings of Kagan et al. (8) corroborated their hypothesis; they found that basal cortisol levels were higher in inhibited young children than in uninhibited ones. An inhibited temperament represents fearful and withdrawn behaviour (9).

However, Feder et al. (10) found evidence for lower night-time cortisol levels in anxious children. These findings can be interpreted in the light of a theory by Gunnar and Vazquez (11), who hypothesized that stressful influence early in life may provoke frequent elevations in cortisol levels, which would eventually lead to down-regulation of components of the HPA-axis. On the basis of this theory, and the assumption that stress in early life is also associated with a risk for anxiety problems (12, 13), one could expect anxiety problems to be associated with relatively low cortisol levels. Taken together, there is still little consensus about whether relatively high or low cortisol levels underlie anxiety problems.

In a previous retrospective study (14), we found that morning cortisol levels were higher in young adolescents with persistent anxiety problems (within ages 4 to 12) than in those with no or only current anxiety problems. However, it is still unclear whether the higher cortisol levels we found in individuals with persistent anxiety problems resulted from long-lasting anxiety problems, or reflected an underlying biological vulnerability that may have caused the anxiety problems.

Better insight in the direction of the association between cortisol measures and anxiety problems would be provided by prospective studies, yet such studies are scarce. Smider et al. (15) found that

higher mean daytime cortisol levels at age 4.5 predicted internalizing problems at age 6. This one prospective study indicates that higher basal daytime cortisol levels at a young age may be a risk factor for internalizing problems across a short time interval.

Many studies in adult patients with major depression indicated that symptoms of depression may cause an association between the broad dimension 'internalizing problems' and HPA-axis activity (11, 16). Hence, it is important to distinguish anxiety from depression when investigating associations between anxiety and HPA-axis activity. To our knowledge, studies that investigated whether cortisol measures specifically predicted anxiety – apart from depression – in young adolescents are lacking.

#### Aims of the study

Our aim was to investigate whether cortisol measures predicted future anxiety, and to compare cortisol values of individuals with persistently high, persistently low, decreasing, or increasing anxiety levels. We hypothesize that: i) high cortisol levels predict high future anxiety levels, ii) morning cortisol levels are highest in individuals with persistently high anxiety levels and iii) cortisol measures would be specifically associated with anxiety, as apart from depression.

#### Material and methods

##### Sample and procedure

This study is part of the TRacking Adolescents' Individual Lives Survey (TRAILS), a prospective cohort study of Dutch young adolescents initially aged 10–12 years old, who are followed biennially, until the age of 24. TRAILS aims to chart and explain the development of mental health from pre-adolescence into adulthood, both at the level of psychopathology, and at the level of underlying vulnerability and environmental risk. This study involves data from the first and second assessment wave of TRAILS, which ran from, respectively, March 2001 to July 2002, and September 2003 to December 2004.

The TRAILS target sample consisted of young adolescents from five municipalities in the North of the Netherlands, including both urban and rural areas. The exclusion criteria were: i) adolescent incapable to participate because of mental retardation or a serious physical illness or handicap and ii) Dutch-speaking parent or parent surrogate not available, and not feasible to administer a part of

the measurements in parent's own language. Of all individuals who were approached ( $n = 3145$ ), 6.7% were excluded. Of the remaining 2935 young adolescents, 76.0% participated in the study ( $n = 2230$ , mean age 11.1 years,  $SD = 0.6$ , 50.8% girls). Participants did not differ from those who refused to participate with respect to the proportion of single parent families, the prevalence of teacher-rated problem behaviour, several sociodemographic variables and mental health outcomes (17). Of the 2230 baseline participants, 96.4% ( $n = 2149$ , 51.0% girls) participated in the second assessment wave, two to three years after wave 1 (mean number of months 29.4,  $SD = 5.4$ , range 16.7–48.1). Mean age at wave 2 was 13.6 ( $SD = 0.5$ ). The Revised Child Anxiety and Depression Scale (RCADS, see below) was completed at the first and second assessment wave by 2081 individuals. At the first wave, we obtained cortisol samples three times on 1 day (sample 1: directly after waking up, sample 2: half an hour later, and sample 3: at 8.00 P.M.) in 1768 participants. Twenty-two participants were excluded because of use of antibiotics or corticosteroids. To reduce the impact of outliers, cortisol values that were above 3  $SD$  of the mean were also excluded. This yielded  $n = 1666$  for sample 1 (21 excluded),  $n = 1683$  for sample 2 (11 excluded), and  $n = 1689$  for sample 3 (18 excluded). All in all, RCADS questionnaire data from both assessment waves and at least one cortisol sample were available for 1623 participants.

To examine possible selective attrition, a stepwise logistic regression analysis was performed in which the 1623 participants with available data for this study were compared with the other individuals who participated at wave 1. Gender, pubertal stage, socioeconomic status, the different cortisol measures (sample, 1, 2, and 3), and scores on the RCADS Total Anxiety and Depression (see below) scales were used as predictors. Low socioeconomic status significantly predicted attrition ( $\beta = 0.68$ ,  $P < 0.00$ ), whereas the other predictors did not. However, the effect size of the entire model was small (Cox and Snell  $R^2 = 1.5\%$ ). After the procedure had been fully explained, written consent was obtained from the young adolescents' parents at both assessments waves. The study was approved by the Central Dutch Medical Ethics Committee.

#### Measures

*Anxiety and co-occurring depressive problems.* The Revised Child Anxiety and Depression Scale [RCADS (18, 19)], a revision of the Spence

Children's Anxiety Scale [SCAS (20)], was used to measure anxiety levels and co-occurring depressive problems. The RCADS is a self-report questionnaire with 47 items that are scored on a 4-point scale (0 = never, 1 = sometimes, 2 = often, 3 = always), and covers symptoms of the following DSM-IV disorders: separation anxiety disorder, generalized anxiety disorder, social phobia, panic disorder, obsessive-compulsive disorder, and major depressive disorder (MDD). In this study, Total Anxiety scores were computed by summing the scores on all the anxiety items and dividing this sum score by the number of items for which answers had been filled in, resulting in a mean score on all anxiety items. If more than 33% of the items were missing, the scale score was coded as missing. Depression (MDD) scores were computed in a similar manner.

The internal consistencies of the scales that were used were – respectively at wave 1/wave 2 – 0.91/0.93 for the Total Anxiety scale, and 0.72/0.81 for the Depression scale. The original factor structure, which was originally based on data from 1641 children and adolescents from a community sample from Hawaii (18), was confirmed by confirmatory factor analysis in the TRAILS sample at wave 1 (fit indices of NNFI=0.96, RMSEA = 0.05, and SRMR = 0.05, indicating an adequate fit to the sample data (21).

*Cortisol.* TRAILS participants collected three samples of saliva with a Salivette sampling device. Collection of cortisol in saliva is a relatively stress-free approach that avoids confounding by stress responses, such as those induced by venipuncture (22). According to several authors, correlations between saliva cortisol levels and serum cortisol concentrations are high (23, 24).

Participants and their parents were instructed to collect saliva at two time points during the morning; directly after waking up (approximately 7.00 A.M., while still lying in bed, sample 1), and half an hour later (sample 2). In addition, saliva was collected at 8.00 P.M. (sample 3). Parents of all participants received written and oral instructions. First, they received a letter containing information about the purpose of saliva collection and some background information about diurnal basal cortisol levels. Then, a member of the team visited them at home and gave further instructions. It was stressed that it was important to collect saliva on a normal day, during a normal week, without special events or stressful circumstances. Parents were also told that their child should not be ill, have a cold, be menstruating, or take any medication on the day of saliva collection. Furthermore, it was

explained that participants should rinse their mouth with tap water before sampling saliva, and not consume sour products or brush their teeth before sampling. Parents were encouraged to place the first salivette next to their child's bed, so that he or she could collect the first sample directly after waking up. In addition, it was stressed that the salivettes should be placed in a freezer directly after saliva collection, and mailed to the institute as soon as possible (but not on Fridays or Saturdays in order to prevent unnecessary delay due to the weekend). Finally, all instructions were also handed out on an instruction form. If any of the requirements were not met, parents could note this down on an accompanying form. Information from this form (e.g. deviant time of sampling) was included in our database. In addition, suspicious quality of the saliva (e.g. containing blood or bread crumbs) was noted by our team in this database. Preliminary analyses showed that these factors were not significantly related to the cortisol levels, and did not differ between the groups that were investigated (individuals with persistently low, decreasing, increasing or persistently high anxiety levels).

Participants who did not return the salivettes within a couple of months were sent a reminder letter. In all, saliva samples of 1768 children (79.3% of all TRAILS participants) were received. For more characteristics of this study population see Rosmalen et al. (25). The saliva samples were stored at  $-20^{\circ}\text{C}$  until analysis. Previous studies suggested that salivary cortisol levels are stable for prolonged periods of time at  $-20^{\circ}\text{C}$  (26). After completion of the data collection, all samples were sent in one batch (frozen, by courier) to the laboratory (Department of Clinical and Theoretical Psychobiology, University of Trier, Germany) for analyses.

Cortisol levels were determined with a competitive solid phase time-resolved fluorescence immunoassay with fluorometric end-point detection (DELFA, dissociation-enhanced lanthanide fluorescent immunoassays). Ninety-six-well Maxisorb microtiterplates (Nunc) were used, coated with rabbit-anti-ovine immunoglobulin. After an incubation period of 48 h at  $4^{\circ}\text{C}$ , the plates were washed with wash buffer ( $\text{pH} = 7.4$ ) coated with an ovine anti-cortisol antibody, and then incubated again. Synthetic saliva mixed with cortisol in a range from 0 to 100 nmol/l served as standards. Standards, controls (saliva pools) and samples were tested in duplicate wells. Fifty microlitres of biotin-conjugated cortisol was added, and after 30 min of incubation the non-binding cortisol/biotin-conjugated cortisol was removed by washing.

Two-hundred microlitres europium-streptavidin (Wallac, Turku, Finland) was added to each well and after 30 min and six times of washing, 200  $\mu\text{l}$  enhancement solution was added (Pharmacia, Freiburg, Germany). Within 15 min on a shaker, the enhancement solution induced fluorescence that could be detected with a DELFIA-Fluorometer (Wallac, Turku, Finland). A standard curve was generated and the cortisol concentrations of the samples were calculated with a computer program. The intra-assay coefficient of variation was between 4.0% and 6.7%, and the corresponding inter-assay coefficients of variation between 7.1% and 9.0% (25).

To obtain an index for the cortisol awakening response, we calculated the area under the curve (AUC) of the two morning cortisol samples (6, 27). The cortisol awakening response is a useful index of HPA-axis activity, which is rather consistent, shows good intra individual stability across time, and appears to be useful for assessing subtle changes in HPA-axis activity (27, 28). Previous research has provided evidence for a significant genetic influence on the cortisol awakening response and this response was found to be independent of the time of awakening, 'manner of awakening' (spontaneously or by an alarm clock), sleep duration, sleep quality, physical activity or morning routines (5, 27, 28). Furthermore, the cortisol awakening response has proved to be a good index for uncovering associations between HPA-axis activity and stress-related problems, such as worrying, social stress, persisting pain and burnout (6). Sample 3 was used to investigate possible associations with evening cortisol levels.

*Other individual characteristics.* Some variables might be associated with both cortisol and anxiety, and might therefore play a confounding role in the relationship between cortisol levels and anxiety levels. In this sample, gender and wave 2 Depression were significantly associated with the index for the cortisol awakening response (AUC) and with wave 2 Total Anxiety scores. Therefore, gender and wave 2 Depression were taken into account as possible confounders.

Information regarding other possible confounders, such as age, pubertal stage, perinatal variables (pregnancy duration, birth weight), body mass index (BMI), and wave 1 and 2 disruptive behaviour was also assessed in the TRAILS study, but was not taken into account for this manuscript, as these factors were not significantly associated with the cortisol measures and with wave 2 Total Anxiety scores, and since addition of these factors to the model did not change the investigated



associations markedly. For the same reasons we did not take into account wave 1 Total Anxiety and Depression scores. Given the age-range of our sample at wave 1 (10–12 years old), there was no use of oral contraceptives (0%), and there was only a very low number of smokers (87% of the selected sample had never smoked, only 1% had smoked seven times or more) at the time of cortisol sampling. Therefore, these variables were also not included in this study.

#### Statistical analyses

The cortisol measures were root-transformed and centered to approximate a normal distribution and to improve the interpretation of betas.

To test whether cortisol measures predicted future anxiety levels, two sets of multiple linear regression analyses were performed with wave 2 Total Anxiety scores as the dependent variable. In the first block of the first set of regression analyses AUC and gender were added as predictors. To investigate possible gender differences in the association between AUC and wave 2 Total Anxiety scores, the interaction between gender and AUC was added as a predictor in the second block. To investigate whether the association was specific for anxiety, wave 2 Depression scores were added as a predictor in the last block. The second set of analyses was performed similarly, except that evening cortisol levels – instead of AUC – and the interaction between evening cortisol levels and gender were added to the model.

As our interest particularly concerned the association between cortisol measures and the persistence of anxiety, we tested whether cortisol values differed between individuals with persistently low, decreasing, increasing, or persistently high anxiety levels. Four groups were composed, using cut-off scores based on the 50th percentile (P50, Total Anxiety = 0.51) and the 80th percentile (P80, Total Anxiety = 0.81) of the cumulative frequency distribution of the wave 1 Total Anxiety scores. The first group, which consisted of individuals with persistently low anxiety levels, scored below P50 on the Total Anxiety scale at wave 1 and at wave 2 ( $n = 642$ ). The second group, with decreasing anxiety levels scored above P80 on the Total Anxiety scale at wave 1 and below P50 on the Total Anxiety scale at wave 2 ( $n = 125$ ). The third group consisted of individuals with increasing anxiety levels, who scored below P50 on the Total Anxiety scale at wave 1, but above P80 on the Total Anxiety scale at wave 2 ( $n = 29$ ). The fourth group consisted of individuals with persistently high anxiety levels who scored above P80 on

the Total Anxiety scale at wave 1 and at wave 2 ( $n = 91$ ). We should note that of those high on Total Anxiety at wave 1, about 50% was also high on Depression at wave 1 (decreasers: 46%, persistently high: 55%). Depression at wave 1 was however not related to the cortisol levels, and was therefore not taken into account as a confounder.

For each group, descriptives were computed. Differences between the groups concerning AUC and evening cortisol levels were calculated using one-way analyses of covariance, with gender and wave 2 Depression scores as covariates. To detect differences between the group with persistently high anxiety levels and the other groups, contrasts were calculated with the group with persistently high anxiety levels as the reference group.

## Results

#### Regression models

The index for the cortisol awakening response (AUC) did not significantly predict wave 2 Total Anxiety scores in the first block ( $B_{auc} = 0.017$ ,  $P = 0.32$ ). Adding the interaction between AUC and gender to the model did not reveal significant gender differences (block 2:  $B_{aucsex} = 0.019$ ,  $P = 0.59$ ). Addition of wave 2 Depression scores did not reveal such gender differences either (block 3:  $B_{aucsex} = -0.017$ ,  $P = 0.47$ ).

Evening cortisol levels also did not significantly predict wave 2 Total Anxiety scores in the first block ( $B_{sample3} = -0.007$ ,  $P = 0.65$ ). Adding the interaction between evening cortisol levels and gender to the model also did not reveal significant gender differences (block 2:  $B_{sample3sex} = -0.024$ ,  $P = 0.45$ ). Addition of wave 2 Depression scores did not reveal such gender differences either (block 3:  $B_{sample3sex} = -0.012$ ,  $P = 0.59$ ).

#### Group differences

Table 1 shows descriptives for each group (persistently low, decreasing, increasing and persistently high anxiety levels). Mean cortisol concentrations throughout the day are shown for each group in Fig. 1.

The one-way analyses of covariance did not reveal an overall difference in AUC between the four groups ( $F(3) = 1.88$ ,  $P = 0.13$ ,  $\eta^2 = 0.7\%$ ), but *post-hoc* contrasts showed that AUC was significantly lower in individuals with persistently high anxiety levels than in those with increasing anxiety levels ( $P = 0.02$ ). Individuals with persis-

Table 1. Descriptives for the groups with different developmental pathways of anxiety (persistently low, decreasing, increasing and persistently high anxiety levels)

Measures	Persistently low anxiety ( <i>n</i> = 642)		Decreasing anxiety ( <i>n</i> = 125)		Increasing anxiety ( <i>n</i> = 29)		Persistently high anxiety ( <i>n</i> = 91)	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Total Anxiety wave 1	0.30 (0.13)	0.00–0.49	1.05 (0.22)	0.81–1.97	0.38 (0.07)	0.22–0.49	1.13 (0.28)	0.81–2.08
Total Anxiety wave 2	0.21 (0.14)	0.00–0.49	0.31 (0.13)	0.00–0.49	0.98 (0.21)	0.81–1.59	1.10 (0.27)	0.81–2.14
Depression wave 1	0.42 (0.24)	0.00–1.20	0.95 (0.34)	0.30–2.10	0.55 (0.27)	0.10–1.10	1.00 (0.34)	0.00–1.90
Depression wave 2	0.23 (0.22)	0.00–1.10	0.38 (0.27)	0.00–1.20	0.92 (0.45)	0.30–2.20	1.02 (0.44)	0.10–2.50
Gender (% girls)	42%	–	45%	–	76%	–	71%	–

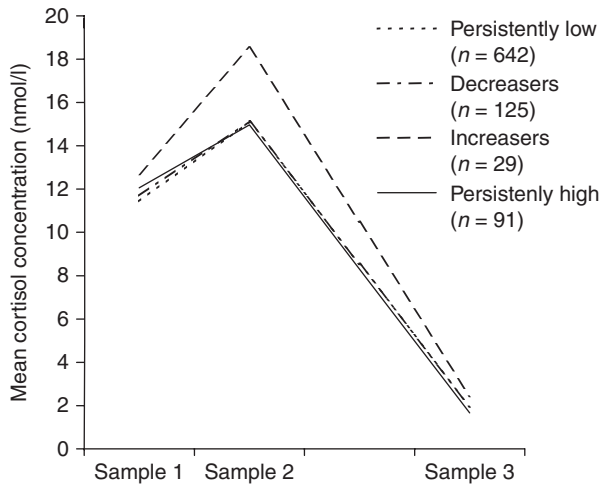


Fig. 1. Mean cortisol concentrations throughout the day for individuals with persistently low, decreasing, or persistently high anxiety levels. Sample 1, directly after waking up; sample 2, half an hour later; sample 3, 8 o'clock in the evening.

tently high anxiety levels did not differ with respect to AUC compared with the other groups (persistently low, decreasing). As Fig. 1 shows, AUC does not only seem to be higher in individuals with increasing anxiety levels compared to those with persistently high anxiety levels, but also compared to those with persistently low or decreasing anxiety levels. We tested this difference for significance by repeating the analyses, using the group with increasing anxiety levels as the reference group. These *post-hoc* analyses revealed that AUC was also higher in individuals with increasing anxiety levels when we compared this group to those with decreasing anxiety levels ( $P = 0.05$ ) or those with persistently low anxiety levels ( $P = 0.05$ ).

The second set of one-way analyses of covariance revealed an overall difference in evening cortisol levels between the four groups ( $F(3)=2.97$ ,  $P = 0.03$ ,  $\eta^2 = 1\%$ ). *Post-hoc* contrasts showed that evening cortisol levels were lower in individuals with persistently high anxiety levels than in those with persistently low ( $P = 0.02$ ), decreasing

( $P = 0.04$ ) or increasing ( $P = 0.01$ ) anxiety levels. These group-differences are also illustrated in Fig. 1.

Finally, Table 1 shows that mean anxiety levels at wave 1 are higher in individuals with increasing anxiety levels than in the group with persistently low anxiety levels. This is the case, while both groups were composed using the same wave 1 cut-off point (below P50 of the Total Anxiety scale). As this difference could be important for the interpretation of our findings, we tested this difference for significance by means of *post-hoc* one-way analysis of covariance. We found that participants with increasing anxiety levels had significantly higher baseline anxiety levels than those with persistently low anxiety levels ( $F(1)=10.47$ ,  $P < 0.01$ ,  $\eta^2 = 1.5\%$ ).

## Discussion

This study investigated the role of the HPA-axis in the development of anxiety in early adolescence. We tested the following hypotheses: i) high cortisol levels predict high anxiety levels two years later, ii) morning cortisol levels (AUC) are higher in individuals with persistently high anxiety levels, than in those with persistently low, decreasing, or increasing anxiety levels, and iii) cortisol measures are specifically associated with anxiety, as apart from depression.

Our analyses revealed that baseline cortisol measures did not predict anxiety levels 2 years later in this general population sample. Moreover, individuals with persistent anxiety problems did not have higher morning cortisol levels (AUC) than those with persistently low, decreasing, or increasing levels of anxiety. Hence, although the TRAILS study provided the opportunity to investigate associations between cortisol measures and future anxiety levels in a large, representative population sample – and thus had the power to detect small effects – the effects we expected were not found. Thus, overall, we conclude that in the general population, cortisol measures alone cannot be used to identify individuals at risk for anxiety problems.

Having said this, two significant group differences were found: The group with increasing anxiety levels had a significantly higher AUC (especially the cortisol levels half an hour after awakening) than the other groups (persistently low, decreasing, persistently high). Further, the group with persistently high anxiety levels had significantly lower evening cortisol levels than the other groups (persistently low, decreasing, increasing). Yet, the sizes of these effects were small.

#### Theoretical considerations

Our findings do not provide clear evidence for either the theory of Kagan et al. (7), who suggest that high cortisol levels may underlie future anxiety problems, or the theory of Gunnar and Vazquez (11) that implies that stress during early development may lead to lower cortisol levels. Having stressed this, our finding of higher morning cortisol levels (especially sample 2) in participants with increasing anxiety levels is noteworthy. In this group with increasing anxiety levels, an increase in anxiety levels apparently involved a higher AUC (i.e. increase in cortisol levels after awakening). This could mean two things. First, it could be that, in this particular group, higher HPA-axis activity, especially after awakening, eventually lead to an increase in anxiety levels, which would be in line with the theory of Kagan et al. (7). This, however, is not very likely, as our regression analyses did not reveal that cortisol measures predicted future anxiety levels. Second, it could be that, in this particular group, an increase in anxiety levels went hand in hand with an increase in cortisol levels after awakening. In other words, as anxiety levels increased, so did these morning cortisol levels. As this group already had somewhat higher baseline anxiety levels than the group with persistently low anxiety levels, this explanation seems more likely. Thus, this finding might indicate that a rise in anxiety levels across time may be reflected in a rise in cortisol levels after awakening.

#### Morning vs. evening cortisol levels

Although the effect size was small, our finding of lower evening cortisol levels in participants with persistently high anxiety levels could be considered as supportive of the theory of Gunnar and Vazquez (11). Persistence of anxiety may first have resulted in higher cortisol levels, and, as a consequence, in down-regulation of components of the HPA-axis in the long run. This may explain the lower evening cortisol levels in those individuals with persistent anxiety. However, only evening cortisol levels were

lower, whereas morning levels were not. Hence, the results do not support the Gunnar and Vazquez theory unequivocally.

Since Feder et al. (10) also found lower evening cortisol levels in anxious children, it might be fruitful to further investigate this phenomenon. Both higher morning cortisol levels and lower evening cortisol levels have been associated with anxiety. Previous research has shown that morning cortisol levels are mainly determined by genetic influences, while evening cortisol levels are mainly influenced by environmental factors (27, 29). Therefore, it is possible that the theories of Kagan et al. (7) and of Gunnar and Vazquez (11) both partly apply, be it for different aspects of HPA-axis functioning.

#### Anxiety vs. depression

Co-occurring depressive problems at wave 2 were taken into account in all analyses. The associations between the cortisol measures and certain developmental trajectories of anxiety (increasing anxiety, persistent anxiety) seemed to be specific for anxiety, because these associations did not disappear when we adjusted for co-occurring depressive problems (wave 2, and preliminary analyses also did not show different results when controlling for wave 1 Depression). Apparently, the associations we found between cortisol and anxiety do not seem to be explained by the broad dimension of internalizing problems, but seem to be quite specific for anxiety, as apart from depression.

#### Previous vs. current findings

One may question why individuals with persistently high anxiety levels did not have relatively high cortisol levels, like we expected based on our previous findings. It could be that, due to down-regulation of components of the HPA-axis, the cortisol levels of individuals with persistent anxiety problems drop again (11). Yet, this does not corroborate with our finding of higher morning cortisol levels (AUC) in individuals with persistent anxiety problems in our previous study (14). This previous study concerned persistency of anxiety problems over a 6–8-year period from early childhood (4 years old) to early adolescence (10–12 years old), after which cortisol levels were obtained. Anxiety levels at age 4 were assessed retrospectively. In the current study, the persistency of anxiety was measured prospectively, and covered a 2-year period of early adolescence. Cortisol levels were assessed at the start of this period. It is possible that the association between



HPA-axis activity and anxiety changes throughout development. Therefore, it is important to investigate this association during different developmental periods. Further, since the period over which persistency of anxiety was measured was longer in the previous study, it might be that the association between cortisol measures and the persistence of anxiety becomes more evident when the persistence of anxiety is measured over a longer period of time. Possibly, cortisol levels increase after a long period of anxiety problems, but high cortisol levels do not predict the persistence of anxiety in the future. Lastly, one should bear in mind that our previous study relied on retrospective data, while the current study was a prospective one, which implies that the reliability of the anxiety data used in the current study was almost certainly higher. Thus, findings might differ between the studies due to some important methodological differences.

#### Methodological considerations

This study was carried out in a general population sample. The theory of Kagan et al. (7) was based on findings in small, select groups and other studies often focused on clinical samples (30). Thus, as anxiety levels are relatively low in our sample, associations between cortisol levels and anxiety might be different in a sample with symptoms in the (sub-)clinical range. We should also note that, although we did control for co-occurring depression, individuals with high Depression scores were not excluded, thus co-occurring depressive problems could have influenced our results to some extent. Further, basal daytime cortisol measures were assessed. Although our index of the cortisol awakening response (AUC) reflects the response to a so-called physical stressor – waking up – measures of reactivity to relatively more stressful stimuli, such as mental stress, might reveal different results. Finally, the present study had some limitations. For instance, cortisol samples were collected at home, which may have limited the reliability of the time points at which cortisol samples were assessed. Fortunately, deviant time of sampling was not significantly related to the cortisol levels. It is however possible that the small effect sizes we found have been influenced by a relatively lower reliability of the cortisol data. Nevertheless, since the effect sizes were particularly small in this study, we may also conclude that anxiety levels in young adolescents are probably associated with many other factors as well. For instance, the HPA-axis works in alliance with many other physiological systems, such as the immune system and the gonadal axis (31),

HPA-axis activity is triggered by environmental influences, and HPA-axis functioning is at least partially determined by genetic factors (27, 29, 32). Therefore, focusing too much on HPA-axis functioning alone in the search for aetiological mechanisms may not prove to be very fruitful.

To conclude, this study did not reveal evidence for a predictive association between baseline cortisol measures and the development of anxiety in young adolescents from the general population. We therefore conclude that in the general population, cortisol measures alone cannot be used to identify individuals at risk for anxiety problems. We question the extent to which the HPA-axis in itself plays an important role in the aetiology of anxiety, and we expect that the interaction of the HPA-axis with other biological or environmental factors will play a more important role.

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#### Declaration of interests

None.

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